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PLANT COLD ACCLIMATION: Freezing Tolerance Genes and Regulatory Mechanisms

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ABSTRACT

Many plants increase in freezing tolerance upon exposure to low nonfreezing temperatures, a phenomenon known as cold acclimation. In this review, recent advances in determining the nature and function of genes with roles in freezing tolerance and the mechanisms involved in low temperature gene regulation and signal transduction are described. One of the important conclusions to emerge from these studies is that cold acclimation includes the expression of certain cold-induced genes that function to stabilize membranes against freeze-induced injury. In addition, a family of Arabidopsis transcription factors, the CBF/DREB1 proteins, have been identified that control the expression of a regulon of cold-induced genes that increase plant freezing tolerance. These results along with many of the others summarized here further our understanding of the basic mechanisms that plants have evolved to survive freezing temperatures. In addition, the findings have potential practical applications as freezing temperatures are a major factor limiting the geographical locations suitable for growing crop and horticultural plants and periodically account for significant losses in plant productivity.

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INTRODUCTION

Over the course of the year, plants from temperate regions vary dramatically in their ability to survive freezing temperatures. In the warm growing seasons, such plants display little capacity to withstand freezing. However, as the year progresses, many sense changes in the environment that signal the coming winter and exhibit an increase in freezing tolerance. The primary environmental factor responsible for triggering this increase in freezing tolerance is low nonfreezing temperatures, a phenomenon known as cold acclimation. Nonacclimated rye, for instance, is killed by freezing at about -5°C, but after a period of exposure to low nonfreezing temperature can survive freezing down to about -30°C.

What genes have important roles in cold acclimation? What are their functions? How do plants sense low temperature and activate the cold-acclimation response? These are some of the key questions that investigators working in the field of cold acclimation are actively engaged in answering. Knowledge in these areas is not only important for an overall understanding of how plants sense and respond to changes in the environment, but also has potential practical applications. Freezing temperatures are a major factor determining the geographical locations suitable for growing crop and horticultural plants and periodically account for significant losses in plant productivity. Determining the nature of the genes and mechanisms responsible for freezing tolerance and the sensing and regulatory mechanisms that activate the cold-acclimation response provide the potential for new strategies to improve the freezing tolerance of agronomic plants. Such strategies would be highly significant as traditional plant breeding approaches have had limited success in improving freezing tolerance (100). The freezing tolerance of wheat varieties today, for instance, is only marginally better than those developed in the early part of this century (16).

The primary purpose of this review is to summarize recent developments regarding the identification of freezing tolerance genes and the regulatory and sensing mechanisms involved in controlling the cold acclimation process. To set the stage for these findings, however, I begin by presenting a brief overview of our current understanding of the causes of freezing injury and mechanisms of freezing tolerance. For additional coverage of these and other aspects of cold

acclimation, the reader is referred to other reviews (27, 42, 43, 117) and books (67, 68, 99).

FREEZING INJURY AND TOLERANCE MECHANISMS

A wide range of studies indicate that the membrane systems of the cell are the primary site of freezing injury in plants (67, 105). In addition, it is well established that freeze-induced membrane damage results primarily from the severe dehydration associated with freezing (105, 108). As temperatures drop below 0°C, ice formation is generally initiated in the intercellular spaces due, in part, to the extracellular fluid having a higher freezing point (lower solute concentration) than the intracellular fluid. Because the chemical potential of ice is less than that of liquid water at a given temperature, the formation of extracellular ice results in a drop in water potential outside the cell. Consequently, there is movement of unfrozen water down the chemical potential gradient from inside the cell to the intercellular spaces. At −10°C, more than 90% of the osmotically active water typically moves out of the cells, and the osmotic potential of the remaining unfrozen intracellular and intercellular fluid is greater than 5 osmolar.

Multiple forms of membrane damage can occur as a consequence of freeze-induced cellular dehydration including expansion-induced-lysis, lamellar-to-hexagonal-II phase transitions, and fracture jump lesions (107, 117). Thus, a key function of cold acclimation is to stabilize membranes against freezing injury. Indeed, cold acclimation prevents expansion-induced-lysis and the formation of hexagonal II phase lipids in rye and other plants (107, 117). Multiple mechanisms appear to be involved in this stabilization. The best-documented are changes in lipid composition (107, 117). However, the accumulation of sucrose and other simple sugars that typically occurs with cold acclimation also seems likely to contribute to the stabilization of membranes as these molecules can protect membranes against freeze-induced damage in vitro (1, 111). In addition, as discussed below, there is emerging evidence that certain novel hydrophilic and LEA (late embryogenesis abundant) polypeptides also participate in the stabilization of membranes against freeze-induced injury.

Although freezing injury is thought to result primarily from membrane lesions caused by cellular dehydration, additional factors may also contribute to freezing-induced cellular damage. There is evidence that freeze-induced production of reactive oxygen species contributes to membrane damage (77) and that intercellular ice can form adhesions with cell walls and membranes and cause cell rupture (90). In addition, there is evidence that protein denaturation occurs in plants at low temperature (25), which could potentially result in cellular damage. In these cases, the enhancement of antioxidative mechanisms

(77), increased levels of sugars in the apoplastic space (73), and the induction of genes encoding molecular chaperones (32), respectively, could have protective effects.

In sum, we have gained important insights into both the causes of freezing injury and mechanisms involved in freezing tolerance. However, our overall understanding of freezing injury and tolerance mechanisms remains far from complete. We are not yet able to design from a core set of principles new wheat varieties that match the freezing tolerance of their close relative, rye, let alone create freezing-tolerant cucumber or banana plants. The identification and characterization of genes that have roles in freezing tolerance should contribute to the development of such "core principles" and potentially provide tools that can be used to improve plant freezing tolerance.

IDENTIFICATION AND CHARACTERIZATION OF FREEZING TOLERANCE GENES

Classical genetic studies have demonstrated that the ability of plants to cold acclimate is a quantitative trait involving the action of many genes with small additive effects [see (114)]. In recent years, three major approaches have been taken to determine the nature of genes with roles in freezing tolerance: the isolation and characterization of genes induced during cold acclimation; the isolation and characterization of mutants affected in freezing tolerance; and QTL mapping using molecular probes to identify freezing-tolerance loci. Recent studies have provided important advances in these areas.

Role of Cold-Regulated Genes in Cold Acclimation

In 1985, Guy et al (33) established that changes in gene expression occur with cold acclimation. Since then, considerable effort has been directed at determining the nature of cold-inducible genes and establishing whether they have roles in freezing tolerance. This has resulted in the identification of many genes that are induced during cold acclimation (see Supplementary Materials Section, http://www.annualreviews.org; Tables 1, 2, and 3 for examples). A large number of these genes encode proteins with known enzyme activities that potentially contribute to freezing tolerance (see Supplementary Materials Section, http://www.annualreviews.org; Table 1). For instance, the *Arabidopsis FAD8* gene (18) encodes a fatty acid desaturase that might contribute to freezing tolerance by altering lipid composition. Cold-responsive genes encoding molecular chaperones including a spinach *hsp70* gene (2) and a *Brassica napus hsp90* gene (58) might contribute to freezing tolerance by stabilizing proteins against freeze-induced denaturation. Also, cold-responsive genes encoding various signal transduction and regulatory proteins have been identified including

a mitogen-activated protein (MAP) kinase (80), a MAP kinase kinase kinase (81), calmodulin-related proteins (95), and 14-3-3 proteins (50). These proteins might contribute to freezing tolerance by controlling the expression of freezing tolerance genes or by regulating the activity of proteins involved in freezing tolerance. Whether these cold-responsive genes actually have important roles in freezing tolerance, however, remains to be determined.

Although many of the genes that are induced during cold acclimation encode proteins with known activities, many do not. Indeed, the largest "class" of cold-induced genes encode polypeptides that are either newly discovered (http://www.annualreviews.org; Table 2) or are homologs of LEA proteins (http://www.annualreviews.org; Table 3), polypeptides that are synthesized late in embryogenesis, just prior to seed desiccation, and in seedlings in response to dehydration stress (9, 13, 44). The polypeptides encoded by the cold-regulated novel and LEA genes fall into a number of families based on amino acid sequence similarities [see (115)]. Interestingly, however, most of these have a set of distinctive properties in common: They are unusually hydrophilic; many remain soluble upon boiling in dilute aqueous buffer; many have relatively simple amino acid compositions, being composed largely of a few amino acids; many are composed largely of repeated amino acid sequence motifs; and many are predicted to contain regions capable of forming amphipathic α-helices. For example:

- *Arabidopsis COR15a* (70) encodes a novel 15-kDa polypeptide that is targeted to the stromal compartment of the chloroplasts ((70); SJ Gilmour & MF Thomashow, unpublished results). The mature 9.4-kDa polypeptide COR15am (Figure 1) is highly hydrophilic; remains soluble upon boiling; is rich is alanine, lysine, glutamic acid, and aspartic acid (they account for 64% of the amino acid residues); and is composed largely of a 13-amino acid sequence that is repeated (imperfectly) four times. Regions of the polypeptide that include the repeated sequences are predicted to form amphipathic α-helices.

- Alfalfa *cas15* (82) encodes a novel 15-kDa polypeptide CAS15 (Figure 1) that is highly hydrophilic; is rich is glutamate, glycine, histidine, and lysine (they account for 68% of the amino acid residues); and nearly a third of the protein is composed of a 10-amino acid sequence that is repeated (imperfectly) four times. Regions of the polypeptide that include the repeated sequences are predicted to form amphipathic α-helices.

- Wheat *wcs120* (41) encodes a 39-kDa polypeptide WCS120 (Figure 1) that is a member of the LEA II group of polypeptides (9). It is highly hydrophilic; remains soluble upon boiling; is rich in glycine, histidine, and threonine

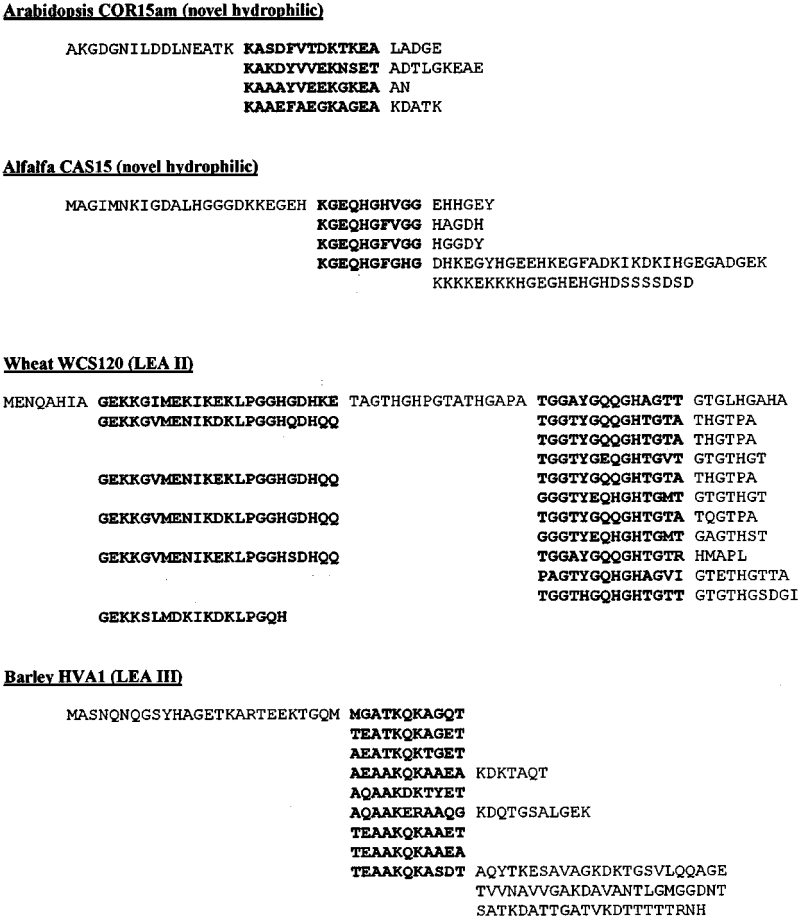

Figure 1 Examples of novel hydrophilic and LEA proteins encoded by genes induced during cold acclimation. The amino acid sequences are presented with the repeated motifs aligned (in bold) to highlight this attribute of each protein. Additional details about the proteins are presented in the text.

residues (they account for 54% of the amino acid residues); and is composed largely of a lysine-rich sequence, referred to as a K-segment, that is repeated (imperfectly) six times and a glycine-rich sequence, referred to as a ϕ-segment, that is repeated (imperfectly) 11 times. The K-segments are present in all LEA II proteins and are predicted to form amphipathic α-helices (9).

- Barley *HVA1* (37, 38) encodes a 22-kDa polypeptide HVA1 (Figure 1) that is member of the LEA III group of proteins (13). It is highly hydrophilic;

rich in alanine, lysine, and threonine (they account for 53% of the amino acid residues); and is composed largely of an 11-amino acid sequence that is repeated (imperfectly) nine times. The repeated sequences are present in all LEA III proteins and are predicted to form amphipathic α-helices (13).

An intriguing possibility regarding the similar biochemical properties shared by many of the novel hydrophilic and LEA polypeptides is that they may reflect some common underlying mechanism of action. Indeed, a possibility discussed in the following section on *Arabidopsis COR* genes is that the amphipathic α-helical regions predicted to be present in many of the novel and LEA proteins may have roles in stabilizing membranes against freezing damage. Whether the regions predicted to form amphipathic α-helices actually form such structures is uncertain (71). Regardless, based on the expression characteristics of the cold-induced novel and LEA genes, namely their induction in response to conditions associated with water deficit—abscisic acid (ABA), drought, high salt, osmotic stress, and seed desiccation—and the close relationship between freezing and dehydration injury, it has often been suggested that these genes might contribute directly to freezing tolerance by protecting cells against the potentially damaging effects of dehydration associated with freezing. Summarized below are recent results obtained with the *Arabidopsis COR* and spinach *CAP* genes that provide direct support for this hypothesis.

ARABIDOPSIS COR GENES The *COR* genes—also designated *LTI* (low temperature-induced), *KIN* (cold-inducible), *RD* (responsive to desiccation), and *ERD* (early dehydration-inducible)—comprise four gene families, each of which is composed of two genes that are physically linked in the genome in tandem array (88, 122, 124–126, 130). At least one member of each gene pair is induced in response to low temperature or other conditions associated with water deficit including drought, high salinity, and ABA. The *COR78* (40, 88, 130), *COR15* (70, 126) and *COR6.6* (59, 60, 122) gene pairs encode newly discovered "boiling soluble" hydrophilic polypeptides (see Supplementary Materials Section, http://www.annualreviews.org; Table 2). The *COR47* gene pair encodes hydrophilic boiling soluble polypeptides that belongs to the LEA II protein family (see Supplementary Materials Section, http://www.annualreviews.org; Table 3), also known as dehydrins and LEA D11 proteins (19, 124, 125).

COR15a expression enhances freezing tolerance Artus et al (4), working with *COR15a*, provided the first direct evidence for a cold-induced gene having a role in freezing tolerance. As mentioned above, *COR15a* encodes a 15-kDa polypeptide that is targeted to the chloroplasts. Upon import into the organelle, COR15a is processed to a 9.4-kDa polypeptide designated COR15am. Artus et al (4) demonstrated that constitutive expression of *COR15a* in nonacclimated

transgenic *Arabidopsis* plants increases the freezing tolerance of both chloroplasts frozen in situ and isolated leaf protoplasts frozen in vitro by 1 to 2°C over the temperature range of −4 to −8°C. In these experiments, it appeared that expression of *COR15a* might also have a slight negative effect on freezing tolerance of protoplasts over the temperature range of −2 to −4°C, but subsequent results by Steponkus et al (106) indicate that this is not the case. It was originally assumed that the protoplasts isolated from the leaves of nonacclimated transgenic plants would have the same intracellular osmolality as those isolated from wild-type plants. In fact, the intracellular osmolality of protoplasts isolated from *COR15a* transgenic plants is approximately .413 osm, whereas that of protoplasts isolated from wild-type plants is about .400 osm (106). The reason for this slight difference is not known. However, when it is taken into account, protoplast survival tests indicate that expression of *COR15a* has only a positive effect on freezing tolerance over the temperature range of −4 to −8°C.

Function of COR15a How does expression of *COR15a* bring about increased freezing tolerance? The results of Artus et al (4) indicated that it involves the stabilization of membranes. This conclusion followed from the fact that protoplast survival was measured using fluorescein diacetate, a vital stain that reports on retention of the semipermeable characteristic of the plasma membrane. The question then became how *COR15a* expression brings about this effect. One possibility was suggested by the work of Steponkus and colleagues (108, 117). These investigators have shown that the formation of hexagonal II phase lipids is a major cause of membrane damage in nonacclimated plants, including *Arabidopsis* (116), over the temperature range of about −4 to −8°C (other forms of membrane damage occur at higher and low temperature due to lesser and greater degrees of dehydration, respectively). Thus, the possibility was that *COR15a* expression might decrease the propensity of membranes to form hexagonal II phase lipids in response to freezing. Indeed, Steponkus et al (106) have found that over the temperature range of −4.5 to −7°C, expression of *COR15a* decreases the incidence of freezing-induced lamellar-to-hexagonal II phase transitions that occur in regions where the plasma membrane is brought into close apposition with the chloroplast envelope as a result of freezing-induced dehydration.

How does COR15am decrease the propensity of membranes to form the hexagonal II phase? More specifically, how can COR15am, which is located in the stromal compartment of the chloroplast, defer to lower temperatures (and thus lower degrees of hydration) the formation of lamellar-to-hexagonal II phase transitions resulting from the interaction of the chloroplast envelope with the plasma membrane? An elegant hypothesis has recently been proposed by Steponkus to explain the phenomenon (106). It is based, in part, on the fact that certain amphipathic α-helices have been shown to have a strong effect on

the intrinsic curvature of monolayers and to affect their propensity to form the hexagonal II phase (14). In brief, Steponkus has proposed that COR15am defers freeze-induced formation of the hexagonal II phase to lower temperatures by altering the intrinsic curvature of the inner membrane of the chloroplast envelope. The onset (the freezing temperature) of hexagonal II phase formation is suggested to be determined by the membrane that has the greatest propensity to form the hexagonal II phase. For the chloroplast envelope-plasma membrane ensemble, there is evidence that the "weak link" is the inner membrane of the chloroplast envelope [see (106)]. Thus, COR15am, which is predicted to have regions that form amphipathic α-helices, is envisioned to alter the intrinsic curvature of the monolayer that comprises the inner membrane of the chloroplast envelope such that its propensity to form the hexagonal II phase is deferred to lower temperatures. A sensitive test of whether a polypeptide has an effect on monolayer curvature is to determine whether the polypeptide causes a shift in the lamellar-to-hexagonal II phase transition temperature. Indeed, Steponkus et al (106) have found that the COR15am polypeptide increases the lamellar-to-hexagonal II phase transition temperature of dioleoylphosphatidylethanolamine and promotes formation of the lamellar phase in a lipid mixture composed of the major lipid species that comprise the chloroplast envelope.

The intrinsic curvature hypothesis advanced by Steponkus provides a possible mechanism for how COR15am increases the freezing tolerance of plant cells. In addition, it may also explain how many of the other novel hydrophilic and LEA polypeptides contribute to increased freezing tolerance. As discussed above, many of these proteins are predicted to contain regions that form amphipathic α-helices. Thus, the intriguing possibility raised is that they too might help stabilize membranes against the dehydration associated with freezing—as well as other environmental conditions such as drought and high salinity—by affecting the intrinsic curvature of membrane monolayers. It will be of interest to determine whether any of the other novel hydrophilic and LEA polypeptides cause a shift in the lamellar-to-hexagonal II phase transition temperature of lipid mixtures. It will also be important to determine whether the regions of the novel and LEA proteins predicted to form amphipathic α-helices actually form such structures (71).

Induction of the CRT/DRE-regulon enhances freezing tolerance Do any other *Arabidopsis COR* genes have roles in freezing tolerance? To address this question, Jaglo-Ottosen et al (49) made transgenic *Arabidopsis* plants that constitutively express the entire battery of *COR* genes and compared the freezing tolerance of these plants to those that expressed *COR15a* alone. Induction of the *COR* genes was accomplished by overexpressing the *Arabidopsis* transcriptional activator CBF1 (CRT/DRE binding factor 1) (109). This factor, which is discussed in greater detail below, binds to the CRT (C-repeat)/DRE

(drought responsive element) DNA regulatory element present in the promoters of the *COR* genes (and presumably other as yet unidentified cold-regulated genes) and activates their expression without a low temperature stimulus (49, 109). What Jaglo-Ottosen et al (49) found was that expression of *CBF1* resulted in a greater increase in freezing tolerance than did expressing *COR15a* alone. In one set of experiments, the electrolyte leakage test (112) was used to assess the freezing tolerance of detached leaves from nonacclimated transgenic and wild-type plants. In this test, plant tissues are frozen to various temperatures below zero degrees Celsius, thawed, and cellular damage is estimated by determining the amount of electrolytes that leach out of the cells, a sign that the plasma membrane has lost its semipermeable characteristic. Jaglo-Ottosen et al (49) did not detect a significant enhancement of freezing tolerance by expressing *COR15a* alone, but detected a 3.3°C increase in freezing tolerance in plants that overexpressed CBF1 and, consequently, the CRT/DRE-regulon of genes. In addition, induction of the CRT/DRE-regulon resulted in an increase in whole plant freezing survival whereas expression of *COR15a* alone did not. Taken together, these results implicate additional cold-regulated genes in freezing tolerance. In addition, given the nature of the electrolyte leakage test, the results indicate that a role of these freezing tolerance genes is to protect membranes against freezing injury.

Liu et al (72) have independently demonstrated that induction of the CRT/DRE-regulon increases the freezing tolerance of *Arabidopsis* plants. In their case, they activated gene expression by overexpressing a homolog of *CBF1*, designated *DREB1A*. Significantly, the results of Liu et al (72) indicate that expression of the CRT/DRE-regulon not only increases freezing tolerance, but also increases tolerance to drought. This finding provides strong support for the notion that a fundamental role of cold-inducible genes is to protect plant cells against cellular dehydration. One additional important finding was that overexpression of *DREB1A* resulted in a dwarf phenotype. This phenotype was not observed by Jaglo-Ottosen et al (49) in plants that overexpress *CBF1*. The reason for this difference is not yet known. It could be due to differences in the level of expression of the transcriptional activators in the two studies. Alternatively, it might be due to differences in the activators used in the experiments. Regardless, the results of both studies provide direct evidence that the CRT/DRE-regulon includes genes that have fundamental roles in cold acclimation.

SPINACH CAP GENES Guy and colleagues have been studying a group of genes, designated *CAP* (cold acclimation protein) (29–31), that are induced during cold acclimation. Two of these, *CAP85* (26, 86) and *CAP160* (26), are also induced in response to dehydration and ABA and encode hydrophilic polypeptides that remain soluble upon boiling. The CAP85 protein belongs to the LEA II group

of proteins; the CAP160 polypeptide is novel, but has a low degree of sequence similarity with the *Arabidopsis* COR78 protein. Both CAP85 and CAP160 are soluble and appear to be located primarily in the cytoplasm, though a portion of CAP160 fractionates with mitochondria, apparently due to an association of the protein with the organelle.

To determine whether the *CAP85* and *CAP160* genes might have roles in freezing tolerance, Kaye et al (54) made transgenic tobacco plants that constitutively expressed the spinach CAP85 and CAP160 proteins and assessed the freezing tolerance of the plants using the electrolyte leakage test. The results suggested that production of the CAP85 and CAP160 proteins, either individually or in combination, had no discernible effect on the freezing tolerance of detached leaves; i.e. the EL_{50} values (temperature that resulted in leakage of 50% of electrolytes) were the same for control and transgenic plants. However, additional experimentation indicated that the proteins slowed the rate of freeze-induced cellular damage; the amount of electrolyte leakage with time of freezing at -2°C was less in the transgenic plants. Thus, both proteins have a detectable effect on freezing tolerance. Again, given the nature of the electrolyte leakage test, it appears that both proteins act to stabilize the plasma membrane against freezing injury. In this regard, it is intriguing that CAP85 is a LEA II protein containing K-segments predicted to form amphipathic α-helices (discussed above) and that regions of CAP160 are predicted to form amphipathic α-helices. Whether these regions of the protein are critical for their apparent activity and whether they affect the intrinsic curvature of membrane monolayers would be interesting to determine.

OTHER POTENTIAL FREEZING TOLERANCE PROTEINS A number of other proteins that accumulate with cold acclimation seem likely to contribute to freezing tolerance (115). These include the cryoprotectin protein of spinach (103), the WSC120 protein family of wheat (101), and the antifreeze proteins that have been described in rye and other cereals (3, 23), bittersweet nightshade (11), and carrot (128). Direct evidence that these proteins have roles in freezing tolerance, however, is not yet available.

Identification of Freezing Tolerance Genes by Mutational Analysis

ARABIDOPSIS SFR GENES Warren and colleagues (78, 123) have used a mutational approach to identify genes in *Arabidopsis* that have roles in freezing tolerance. They screened M_3 seed pools, derived from 1804 chemically mutagenized M_2 plants, for lines that displayed no adverse effects during the cold acclimation treatment (i.e. did not display a chilling-sensitive phenotype that may have indirectly affected freezing tolerance), but did not attain normal levels of freezing tolerance. These efforts resulted in the identification of five

SFR (sensitivity to freezing) genes that appear to have significant roles in cold acclimation: *SFR1, 2, 4, 5,* and *6*. Whereas wild-type *Arabidopsis* seedlings that are cold-acclimated for 2 weeks at 4°C suffer no obvious damage upon being frozen at −6°C for 24 h (followed by incubation at normal growth temperature), seedlings carrying the *sfr1, 2, 4, 5*-1, *5*-2, and *6* mutant alleles do, the nature of which varies with the mutation. The *sfr1* mutation affects the freezing tolerance of only young leaves; the *sfr6* mutation has its most severe effects on young leaves, but affects all leaves to some extent; and the *sfr2, 4, sfr5*-1, and *sfr5*-2 mutations affect all leaves equally. Significantly, all of the mutations affect the cryostability of the plasma membrane, as indicated by the electrolyte leakage test. With the *sfr1, 4, 5,* and *6* mutations, the severity of the freezing damage observed in the whole plant freezing tests corresponds with the results of the electrolyte leakage test. Thus, the freezing sensitivity caused by these mutations appears to result largely from a decrease in membrane cryostability. In contrast, the *sfr2* mutation results in severe injury in the whole plant freeze test, but only minor damage in the electrolyte leakage test. Thus, the freezing-sensitive lesion caused by this mutation might not have a primary effect on cellular membranes.

Determining the nature and functions of the *SFR* genes should provide significant new insight into our understanding of the cold-acclimation response. Indeed, the *sfr2* and *sfr5* mutations do not have any obvious effects on the alterations in fatty acid composition or the increases in sucrose and anthocyanin levels that normally occur with cold acclimation. Thus, the study of these genes may lead to the discovery of freezing-tolerance mechanisms that have not yet been considered. In contrast, the *sfr4* mutation results in reduced accumulation of sucrose, glucose, and anthocyanin and lowered levels of 18:1 and 18:2 fatty acids. Given the likely role of sugars as cryoprotectants and roles of fatty acid composition in membrane cryostability, it is reasonable to speculate that the effects that the *sfr4* mutation have on sugar and fatty acid composition account, at least in part, for the freezing-sensitive phenotype of these mutants. How the *sfr4* mutation brings about such pleiotropic effects, however, is less clear. An interesting possibility, however, is that the *SFR4* gene may have a role in regulating the activation of cold acclimation.

ARABIDOPSIS ESKIMO1 GENE Xin & Browse (129) have also used a mutational approach to identify *Arabidopsis* genes with important roles in cold acclimation. In their case, the investigators screened 800,000 chemically mutagenized M_2 seedlings for mutants that displayed "constitutive" freezing tolerance; i.e. mutants that were more freezing tolerant than wild-type plants without cold acclimation. This resulted in the identification of a gene, *eskimo1* (*esk1*), that has a major effect on freezing tolerance. Whereas nonacclimated wild-type

plants had an LT_{50} of $-5.5^{\circ}C$ in a whole-plant freeze test, nonacclimated *esk1* mutant plants had an LT_{50} of $-10.6^{\circ}C$. Moreover, the *esk1* mutation increased the freezing tolerance of cold-acclimated plants. Wild-type plants that had been cold-acclimated had an LT_{50} of $-12.6^{\circ}C$, while cold-acclimated *esk1* plants had an LT_{50} of $-14.8^{\circ}C$.

The molecular basis for the increase in freezing tolerance displayed by the *esk1* mutation is not yet certain. However, the concentration of free proline in the *esk1* mutant was found to be 30-fold higher than in wild-type plants. It seems likely that this dramatic increase in proline contributes to the increased freezing tolerance of the *esk1* plants as proline has been shown to be an effective cryoprotectant in vitro (7, 98). In addition, total sugars are elevated in the *esk1* mutant about twofold and expression of the *RAB18* cold-responsive LEA II gene is elevated about threefold. These alterations may also contribute the increase in freezing tolerance. Significantly, the *esk1* mutation does not appear to affect expression of the *COR* genes; the transcript levels for *COR15a*, *COR6.6*, *COR47*, and *COR78* remained at low levels under normal growth conditions in the *esk1* plants and were greatly induced in response to low temperature. Xin & Browse (129) suggested that these results may mean that there are multiple signaling pathways involved in activating different aspects of the cold-acclimation response and that activation of one pathway may result in considerable freezing tolerance without activation of the other pathways. As discussed above, overexpression of the CBF1 transcription factor induces expression of the CRT/DRE-regulon and results in a significant increase in freezing tolerance. The "CBF1 pathway" might, therefore, control one set of cold-acclimation responses. Similarly, the *ESK1* gene may participate in the control of another set of freezing tolerance responses that includes synthesis of proline, and to a lesser degree, the synthesis of sugars and expression of *RAB18*. The mechanism of *ESK1* action is not known. However, the fact that the two available *esk1* alleles are recessive suggests that *ESK1* may act as a negative regulator (129).

Mapping Freezing Tolerance Genes—The Wheat Vrn1-Fr1 Interval

The ability of plants to cold acclimate is a quantitative trait [see references in (114)]. Indeed, in wheat, there is evidence that nearly all chromosome pairs can contribute to freezing tolerance. Recent studies have identified a locus on chromosome 5A, the *Vrn1-Fr1* interval, that has a major effect on freezing tolerance (17).

The *Vrn1-Fr1* interval contains the *Vrn1* gene, a major determinant of growth habit (6, 96, 104). Winter-type plants, which are sown in autumn, carry recessive *vrn1* alleles. Such plants require a period of vernalization (exposure to low temperature) to promote floral development. The vernalization requirement is

thought to have evolved to insure that overwintering plants do not flower before the warm growing season. In contrast, spring-type plants can be sown in spring as they carry dominant *Vrn1* alleles that allow floral development without vernalization. Significantly, winter-type plants carrying *vrn1* alleles are almost exclusively more freezing tolerant than spring-type plants carrying *Vrn1* alleles, which indicates that either *Vrn1* itself is a freezing tolerance gene(s) or that it is tightly linked to a freezing tolerance gene(s). The results of Galiba et al (17) support the latter possibility. These investigators analyzed the progeny from a cross between substitution lines of 'Chinese Spring' (a spring-type wheat) carrying 5A chromosomes from either 'Cheyenne', a freezing-tolerant winter wheat or a freezing-sensitive spring-type accession of *Triticum spelta*. Among the progeny they found a single recombinant line that carried the *vrn1* allele (i.e. was a winter-type) but was freezing sensitive. The freezing tolerance gene linked to *Vrn1* was designated *Fr1*.

Additional important information about the *Vrn1-Fr1* interval has come from a study by Storlie et al (110). These investigators addressed the question of whether differences in freezing tolerance among winter wheat varieties involved differences at the *Vrn1-Fr1* interval. This was accomplished by examining the freezing tolerance of near isogenic lines (NILs) carrying *Vrn1-Fr1* intervals from different varieties. Specifically, NILs were derived from five back-crosses between 'Marfed', a freezing-sensitive (LT_{50}–8.2°C) spring wheat that was used as the recurrent parent, and two winter wheat donor parents that differed in freezing tolerance, 'Suweon 185' (LT_{50}–13.6°C) and 'Chugoku 81' (LT_{50}–12.7°C). An analysis of the progeny indicated that those carrying the winter *vrn1-Fr1* locus were about 4°C more freezing tolerant than those carrying the spring *Vrn1-fr1* locus. Also, progeny carrying the *vrn1-Fr1* locus from 'Suweon 185' were about 0.5°C more freezing tolerant than those carrying the *vrn1-Fr1* locus from 'Chugoku 81'. The *Vrn1-Fr1* interval accounted for 70 to 90% of the difference in the freezing tolerance of the NILs, substantiating the importance of this locus in cold acclimation. In addition, the results indicate that differences in freezing tolerance between winter cultivars can, in at least some cases, result from differences at this locus.

The mechanism whereby the *Vrn1-Fr1* interval affects freezing tolerance remains to be determined. Limin et al (69) have shown that cold-induced expression of the *wcs120* genes, which are located on chromosomes 6A, 6B, and 6C, is higher in a winter-type 'Chinese Spring' ('Cheyenne' 5A) substitution line than in the spring-type parent 'Chinese Spring'. In addition, the freezing tolerance of the 'Chinese Spring' ('Cheyenne' 5A) line is greater than that of 'Chinese Spring'. Thus, the possibility raised is that the *Vrn1-Fr1* interval encodes a protein(s) involved in regulating the expression of cold-inducible genes that have roles in freezing tolerance.

A final point regards conservation of the *Vrn1-Fr1* interval in plants. QTL mapping in barley using a cross between the winter variety 'Dicktoo', which is relatively freezing tolerant, and the spring variety 'Morex', which is relatively freezing sensitive, has resulted in the identification of a 21-cM region on chromosome 7 that has a major role in freezing tolerance (35, 93, 119). This region accounted for 32% of the variance in LT_{50} freezing tolerance values and 39–79% of the variance in winter field survival observed in the population. In addition, the region accounted for 47% of the variation for the winter-spring growth habit. Thus, this region encodes major genes for both freezing tolerance and vernalization. Significantly, these genes are likely to correspond to those included in the *Vrn1-Fr1* interval of wheat. This is suggested by the findings that chromosome 7 of barley is homologous to the group 5 chromosomes of wheat (48); the Xwg644 and Xcdo504 molecular markers that are linked to the *Vrn1-Fr1* locus of wheat are contained within the 21-cM freezing tolerance interval of barley [see discussion in (17)]; and the *Sh2* vernalization locus of barley is linked to the Xwg644 marker (65). Whether the freezing tolerance gene(s) contained within the *Vrn1-Fr1* interval is present outside of cereals remains to be determined.

REGULATION OF THE COLD ACCLIMATION RESPONSE

Current evidence suggests that multiple mechanisms are involved in activating the cold-acclimation response. As discussed above, the *Arabidopsis eskimo1* gene (129) appears to have a role in regulating freezing tolerance that is independent of the mechanism that regulates expression of the *Arabidopsis* freezing tolerance CRT/DRE-regulon. Moreover, cold-regulated gene expression itself involves multiple mechanisms including transcriptional (5, 40, 121, 130) and post-transcriptional (12, 94) processes and both "ABA-dependent" (64, 88) and "ABA-independent" pathways (21, 87, 124, 131). In addition, the changes in lipid composition and accumulation of sugars that are likely to contribute to freezing tolerance do not necessarily rely on changes in gene expression, but may be brought about, at least in part, by alterations in the activities of enzymes involved in their synthesis. A complete understanding of how the cold-acclimation response is activated by low temperature will require considerable effort. However, significant insights have begun to emerge.

The CBF/DREB1 Regulatory Genes

There is direct evidence that the *Arabidopsis COR* genes have roles in cold acclimation (49, 72). Thus, understanding how these genes are activated by low temperature should reveal at least one pathway important in regulating

the cold-acclimation response. Toward this end, recent studies have led to the identification of the *CBF/DREB1* family of regulatory genes, "master-switches" (100) involved in *COR* gene induction and cold acclimation.

REGULATION OF COR GENES BY THE CBF/DREB1 TRANSCRIPTIONAL ACTIVATORS

Gene fusion studies have demonstrated that the promoters of the *Arabidopsis COR15a* (5), *COR6.6* (121), and *COR78* (40, 130) genes are induced in response to low temperature. The cold-regulatory element that appears to be primarily responsible for this regulation was first identified by Yamaguchi-Shinozaki & Shinozaki (131) in their study of the *RD29A* (*COR78*) promoter. It is a 9-bp element, TACCGACAT, referred to as the DRE (dehydration responsive element). The DRE, which has a 5-bp core sequence of CCGAC designated the CRT (C-repeat) (5), stimulates gene expression in response to low temperature, drought, and high salinity, but not exogenous application of ABA (131). The element is also referred to as the LTRE (low temperature regulatory element) (51, 88).

Stockinger et al (109) isolated the first cDNA for a protein that binds to the CRT/DRE sequence. The protein, designated CBF1 (CRT/DRE binding factor 1), has a mass of 24 kDa, a putative bipartite nuclear localization sequence, and an acidic region that potentially serves as an activation domain. In addition, it has an AP2 domain, a 60-amino acid motif that has been found in a large number of plant proteins including *Arabidopsis* APETALA2 (52), AINTEGUMENTA (55), and TINY (127); the tobacco EREBPs (ethylene response element binding proteins) (89); and numerous other plant proteins of unknown function [see (97)]. Ohme-Takagi & Shinshi (89) have demonstrated that the AP2 domain includes a DNA-binding region. Interestingly, Stockinger et al (109) noted that the tobacco ethylene response element, AGCCGCC, closely resembles CRT/DRE sequences, GGCCGAC and TACCGAC, present in the promoters of *Arabidopsis COR15a* and *COR78*, respectively. Thus, Stockinger et al (109) suggested that CBF1, the EREBPs, and perhaps other AP2 domain proteins may be members of a superfamily of DNA binding proteins that recognize a family of *cis*-acting regulatory elements that have CCG as a common core sequence. Differences in the sequence surrounding the CCG core element were suggested to result in recruitment of distinct AP2 domain proteins that are integrated into signal transduction pathways activated by different environmental, hormonal, and developmental cues.

The CBF1 protein binds to the CRT/DRE sequence and activates expression of reporter genes in yeast carrying the CRT/DRE as an upstream regulatory sequence (109). These results indicated that CBF1 is a transcriptional activator that can activate CRT/DRE-containing genes and, thus, was a probable regulator of *COR* gene expression in *Arabidopsis*. Indeed, as discussed above,

Jaglo-Ottosen et al (49) have shown that constitutive overexpression of *CBF1* in transgenic *Arabidopsis* plants results in expression of CRT/DRE-controlled *COR* genes without a low temperature stimulus. Thus, CBF1 appears to be an important regulator of the cold-acclimation response, controlling the level of *COR* gene expression, which, in turn, promotes freezing tolerance.

The results of Stockinger et al (109) and Jaglo-Ottosen et al (49) have recently been extended by Gilmour et al (22), Liu et al (72), and Shinwari et al (102). These investigators have established that *CBF1* is a member of a small gene family encoding three closely related transcriptional activators. The three genes, referred to as either *CBF1*, *CBF2*, and *CBF3* (22) or *DREB1B*, *DREB1C*, and *DREB1A*, respectively (72, 102), are physically linked in direct repeat on chromosome 4 near molecular markers PG11 and m600 (∼71 cM) (22, 102). They are unlinked to their target CRT/DRE-controlled genes, *COR6.6*, *COR15a*, *COR47*, and *COR78*, which are located on chromosomes 5, 2, 1, and 5, respectively (22). Like CBF1, both the CBF2 and CBF3 proteins can activate expression of reporter genes in yeast that contain the CRT/DRE as an upstream activator sequence, indicating that these two family members are also transcriptional activators (22). Indeed, Liu et al (72) have shown that overexpression of *DREB1A/CBF3* in transgenic *Arabidopsis* plants results in constitutive expression of *RD29A* (*COR78*) and, as described above, enhances both the freezing and drought tolerance of the transgenic plants.

LOW TEMPERATURE REGULATION OF THE CBF/DREB1 GENES The transcript levels for all three *CBF/DREB1* genes increase dramatically within 15 min of transferring plants to low temperature, followed by accumulation of *COR* gene transcripts at about 2 h (22, 72). Thus, Gilmour et al (22) suggested that *COR* gene expression involves a low-temperature signaling cascade in which *CBF* gene expression is an early step. Regulation of the *CBF/DREB1* genes appears to occur, at least in part, at the transcriptional level as hybrid genes containing the *CBF/DREB1* promoters fused to reporter genes are induced at low temperature (102; D Zarka, M Thomashow, unpublished results). As noted by Gilmour et al (22), the fact that *CBF* transcripts begin accumulating within 15 min of plants being exposed to low temperature strongly suggests that the low-temperature "thermometer" and "signal transducer" are present at warm noninducing temperatures. Gilmour et al (22) have, therefore, proposed that there is a transcription factor already present at warm temperature that recognizes the *CBF* promoters. This factor would not appear to be the CBF proteins themselves as the promoters of the *CBF* genes lack the CRT/DRE sequence and overexpression of *CBF1* does not cause accumulation of *CBF3* transcripts (22). Gilmour et al (22) have, therefore, proposed that *COR* gene induction involves a two-step cascade of transcriptional activators in which the first step,

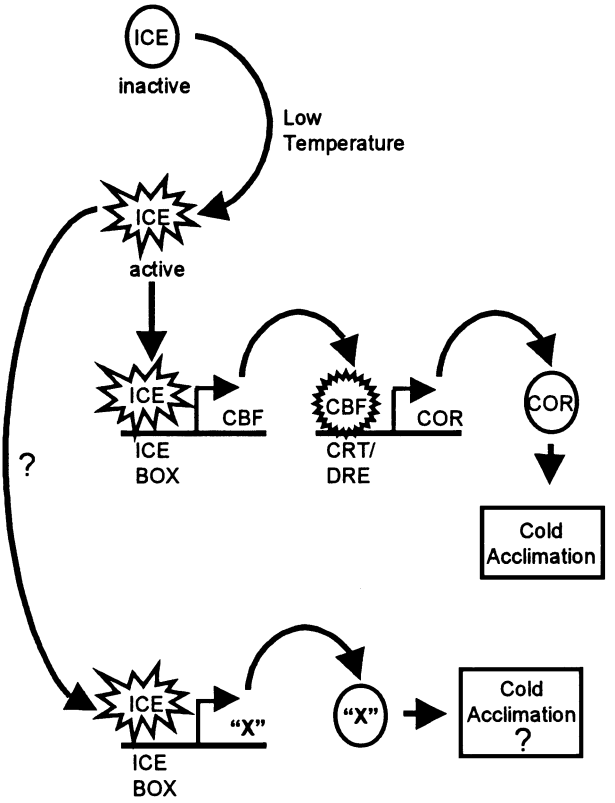

Figure 2 Model for *CBF* regulation of *COR* gene expression (22). See text for details. Reprinted by permission of *The Plant Journal*, 1999.

CBF induction, involves an unknown activator that they tentatively designated "ICE" (inducer of CBF expression) (Figure 2). ICE presumably recognizes a cold-regulatory element, the "ICE Box," present in the promoters of each *CBF* gene. At warm temperature, ICE is suggested to be in an "inactive" state, either because it is sequestered in the cytoplasm by a negative regulatory protein or is in a form that does not bind to DNA or does not activate transcription effectively. Upon exposing a plant to low temperature, however, a signal transduction pathway is suggested to be activated that results in modification of either ICE or an associated protein, which, in turn, allows ICE to induce *CBF* gene expression. As noted by Gilmour et al (22), it is possible that ICE may not only regulate the expression of the *CBF* genes, but might induce expression of other genes ("X") that may also have roles in cold acclimation.

As previously mentioned, the CRT/DRE not only imparts cold-regulated gene expression, but also dehydration-regulated gene expression. The fact that the *CBF* genes are up-regulated in response to low temperature is consistent with the CBF proteins acting at the CRT/DRE to induce expression of *COR* and other cold-regulated CRT/DRE-controlled genes. However, the results of both Gilmour et al (22) and Liu et al (72) indicate that the *CBF* genes are not induced significantly in response to dehydration stress. How then does the CRT/DRE impart dehydration-induced gene expression? Liu et al (72) have provided an answer for this question. They have identified two additional genes, *DREB2A* and *DREB2B*, that are induced in response to dehydration stress and encode proteins that bind to the CRT/DRE. The DREB2 proteins have AP2 domains that are very similar in sequence to those found in the CBF/DREB1 proteins. Outside of the AP2 domains, however, the DREB2 and CBF/DREB1 proteins share little sequence similarity. Liu et al (72) have proposed that induction of the *DREB2* genes leads to the synthesis of DREB2 proteins that bind to the CRT/DRE and activates gene expression. Interestingly, unlike overexpression of the *CBF* genes that results in constitutive expression of the CRT/DRE-controlled *COR* genes, constitutive overexpression of *DREB2A* has only a minor effect on expression of *COR78*. Thus, Liu et al (72) have suggested that the *DREB2* proteins are likely to be activated posttranslationally in response to dehydration stress.

CONSERVATION OF THE CBF/DREB1 REGULATORY PATHWAY An important question is whether the CBF/DREB1 regulatory pathway is highly conserved in plants. The available data are sketchy on this point, but lend support to the notion that it is. In particular, Singh and colleagues (51) have shown that the promoter of the cold-regulated *Brassica napus BN115* gene (an ortholog of *Arabidopsis COR15a*) is induced in response to low temperature and that this induction is dependent on the action of CRT-containing LTREs present within the promoter. Similarly, Sarhan and colleagues have shown that the cold-regulated *wcs120* gene of wheat has a cold-inducible promoter with two putative CRT/LTREs (120), and have presented results of a deletion analysis that are consistent with the CRT/LTREs having a role in low-temperature induction of the promoter (92). In addition, these investigators have presented results indicating that the *Wcs120* promoter is cold-inducible in the monocotyledonous plants barley, rye, and rice, as well as the dicotyledonous plants alfalfa, *Brassica*, and cucumber (though not in tomato and pepper) (92).

Role of ABA

In 1983, Chen et al (8) reported that ABA levels increase transiently in response to low temperature in *Solanum commersonii*, a plant that cold acclimates, but

not in *S. tuberosum*, a plant that does not cold acclimate. Moreover, they found that exogenous application of ABA increases the freezing tolerance of *S. commersonii* plants at warm temperatures. These findings led Chen and colleagues to hypothesize that cold acclimation is activated through the action of ABA; i.e. that low temperature brings about an increase in ABA, which triggers the activation of freezing tolerance mechanisms. Subsequent studies extended the observations of Chen et al (8), establishing that ABA levels increase, at least transiently, in a diverse group of plant species during cold acclimation (28, 62, 63, 74) and that exogenous application of ABA at warm nonacclimating temperature enhances the freezing tolerance of several plant species that cold acclimate (8, 45, 91).

If ABA has a critical role in activating the cold-acclimation response, then one would expect that the freezing tolerance of plants carrying mutations in ABA synthesis or the ability to respond to ABA would be less than that of wild-type plants. This has been shown to be the case in *Arabidopsis*. Seedlings carrying either the *aba1* (formerly *aba*-1) or *abi1* mutations that, respectively, result in impairment of ABA synthesis and insensitivity to ABA [see (57)], are less freezing tolerant than wild-type plants (21, 36, 75); *ABA1* encodes a zeaxanthin epoxidase (76) and *ABI1* encodes a phosphatase 2C (66, 79). These results have led some to conclude that ABA does indeed have a key role in activating cold acclimation. However, the increase in ABA that occurs in response to low temperature is transient in *Arabidopsis*—ABA levels peak at 24 h and return to essentially normal levels by two days (63)—yet freezing tolerance continues to increase for about a week and remains elevated for at least three weeks (20, 61, 116). Moreover, although the *aba1* and *abi1* mutations result in a decrease in *Arabidopsis* freezing tolerance, these mutations also have pleiotropic effects. Plants carrying either the *aba1* or *abi1* mutation display a wilted phenotype and have reduced vigor, for instance. Thus, Gilmour & Thomashow (21) cautioned that the decrease in freezing tolerance caused by the ABA mutations might not be a direct consequence of ABA having a fundamental role in activating cold-acclimation mechanisms, but instead, might be an indirect effect of ABA having important integral roles in plant growth and development. Simply put, "sick" plants might not be able to cold acclimate as well as "healthy" plants.

If ABA does not have a role in activating the cold-acclimation response, why would its exogenous application increase the freezing tolerance of *Arabidopsis* and other plants? A possible explanation is offered by considering the regulation of *Arabidopsis COR* genes. As discussed above, expression of the *Arabidopsis* CRT/DRE-regulon at normal growth temperatures results in an increase in plant freezing tolerance (49, 72). Significantly, at least some of the genes in this regulon, including members of the *COR* gene family—*COR78*,

COR47, *COR15a*, and *COR6.6*—are highly expressed in response to exogenous application of ABA (19, 34, 60, 88, 130). The activation of these genes by exogenous ABA is consistent with observing an increase in freezing tolerance.

Does ABA have an important role in activating expression of CRT/DRE-controlled genes during cold acclimation? There are indications that it does not. It has been shown that cold-induced expression of *COR78*, *COR47*, and *COR6.6* is essentially normal in plants carrying the *aba1* mutation (21, 87). Moreover, the *abi1* mutation essentially abolishes ABA-induced accumulation of transcripts for *COR78*, *COR47*, and *COR6.6*, but has little or no effect on cold-induced accumulation of these transcripts (21, 87). Thus, both Nordin et al (87) and Gilmour & Thomashow (21) proposed that cold-regulated expression of these genes occurs through an ABA-independent pathway. The discovery of the DRE DNA regulatory element by Yamaguchi-Shinozaki & Shinozaki (131) seemingly proved this hypothesis correct. As mentioned above, the element, which is present in the promoters of *COR78*, *COR47*, *COR15a*, and *COR6.6*, imparts cold-regulated gene expression, but does not stimulate transcription in response to exogenous application of ABA. Recently, however, the notion of an ABA-independent pathway regulating CRT/DRE-controlled *COR* genes has been challenged by Zhu and colleagues (47). These investigators have reported the isolation of *Arabidopsis* mutants that "hyper-express" *COR78* in response to both cold and ABA, as well as mutants that are diminished in their expression of *COR78* in response to both cold and ABA. Thus, Zhu and colleagues have proposed that cold and ABA regulatory pathways are not completely independent, but instead have points at which they "cross-talk." Thus, it is not yet certain whether cold-regulated expression of the freezing tolerance CRT/DRE-regulon is completely independent of ABA.

Is cold-regulated expression of any gene dependent on the action of ABA? This appears to be the case for the *Arabidopsis RAB18* and *LTI65* genes; cold-induced accumulation of transcripts for these genes is severely impaired in plants carrying either the *aba1* or *abi1* mutations (64, 88, 124). ABA-regulated expression of these genes is presumably mediated through the action of the putative ABA-responsive elements (ABREs) (24) present in the promoters of these genes (64, 88, 131). As has been shown for other ABA-regulated genes, bZIP transcription factors are likely to bind to these elements in cold-regulated genes and activate gene expression (15). The induction of both *RAB18* and *LTI65* in response to low temperature, however, is very weak. Indeed, one study concluded that *LTI65* (*RD29B*) is not responsive to low temperature at all (131). In contrast, both of these genes are highly responsive to exogenous application of ABA and to dehydration stress (64, 88, 131). The dramatic difference in the relative responses of these genes to drought and low temperature is not surprising, however, as ABA levels are induced to much higher levels in

drought-stressed plants than they are in cold-treated plants (63). Whereas low temperature brings about a transient three- to fourfold increase in ABA content, drought stress brings about more than a 20-fold increase in ABA levels (63).

In sum, the issue of whether ABA has a fundamental role in activating the cold-acclimation response beyond its "general" role in plant growth and development is unresolved. The available evidence seems to suggest that ABA has a relatively minor role in inducing the expression of genes in response to low temperature, but firm conclusions even in this regard are probably premature. A final resolution of the role of ABA in activating the cold-acclimation response will require a more detailed understanding of the specific mechanisms that have roles in freezing tolerance and a determination of whether ABA has a critical role in regulating the activity of these mechanisms.

Low Temperature Signal Transduction

ROLE OF CALCIUM There is mounting evidence that calcium is an important second messenger in a low temperature signal transduction pathway involved in regulating the cold-acclimation response (56, 83, 85, 113). In both *Arabidopsis* (56, 95) and alfalfa (83), cytoplasmic calcium levels increase rapidly in response to low temperature, due largely to an influx of calcium from extracellular stores. Through the use of chemical and pharmacological reagents, it has been shown that this increase in calcium is required for full expression of at least some cold-regulated genes, including the CRT/DRE-controlled *COR6.6* and *KIN1* genes of *Arabidopsis*, and for plants to increase in freezing tolerance (56, 83, 85, 113). For instance, Dhindsa and colleagues (83, 85) have shown that in alfalfa, calcium chelators such as BAPTA (1,2-*bis*(*o*-aminophenoxy)ethane *N*, *N*, *N*′, *N*′-tetraacetic acid) and calcium channel blockers such as La^{3+} inhibit cold-induced influx of calcium and cause both decreased expression of the cold-inducible *cas15* gene and block the ability of alfalfa to cold acclimate. In addition, they have shown that *cas15* expression can be induced at 25°C by treating cells with A23187, a calcium ionophore that causes a rapid influx of calcium.

Two important issues now are to identify the nature of the channels that are presumably responsible for the influx of calcium that occurs with low temperature in *Arabidopsis* and alfalfa and to determine the steps between calcium influx and the activation of gene expression and cold acclimation. In regard to channels, it is of interest that onion has been reported to have a mechanosensitive calcium-selective cation channel that is activated in response to low temperature (10). As for the steps in signal transduction following calcium influx, little is known. However, recent results strongly suggest that protein phosphorylation is involved. In particular, Monroy et al (84) have shown that low temperature induction of alfalfa *cas15* is inhibited by the protein kinase inhibitor staurosporine

and is induced at 25°C by the protein phosphatase inhibitor okadaic acid. Moreover, they have found that low temperature causes a rapid and dramatic decrease in protein phosphatase 2A (PP2A) activity and that this is dependent on calcium influx. Taken together, these results suggest that low temperature leads to an influx in calcium, which inhibits PP2A activity, and that this, in turn, leads to the phosphorylation of one or more proteins involved in inducing *cas15* expression and activating cold acclimation.

The protein kinase(s) responsible for inducing the expression of cold-regulated genes and activating freezing tolerance mechanisms is not known. There are, however, a number of interesting candidates. One is a mitogen-activated protein (MAP) kinase described by Jonak et al (53). This kinase, designated $p44^{MMK4}$, is activated within 10 min of alfalfa plants being exposed to low temperature. Significantly, two other alfalfa MAP kinases, MMK2 and MMK3, are not activated by low temperature, indicating that there is specificity in cold activation of MAP kinases (53). It is also interesting that the transcript levels for $p44^{MMK4}$ increase rapidly (within 20 min) in response to low temperature (though the amount of $p44^{MMK4}$ protein does not change). Indeed, the transcript levels for a number of protein kinases have been shown to increase in response to low temperature. In *Arabidopsis*, genes encoding a MAP kinase kinase kinase, an S6 ribosomal protein kinase, and a MAP kinase are simultaneously induced in response to low temperature, as well as touch and dehydration stress (81). In addition, there is evidence that the transcript levels for calcium-dependent protein kinases (CDPKs) in *Arabidopsis* (113) and alfalfa (83) accumulate in response to low temperature, as do transcripts for an *Arabidopsis* receptor-like protein kinase (39) and two *Arabidopsis* two-component response regulator-like proteins (118). At present, all of these kinases would seem to be candidates for having roles in cold acclimation.

ROLE OF ARABIDOPSIS HOS1 GENE As alluded to earlier, Zhu and colleagues (47) have described the isolation of *Arabidopsis* mutants altered in cold-regulated gene expression. One mutation identified, *hos1*-1, alters the temperature at which the *RD29A* promoter becomes activated; in wild-type plants, the promoter is essentially inactive until the temperature falls below 10°C, whereas low-level induction of the promoter can be detected in the *hos1*-1 mutant even at 19°C (46). Moreover, the *hos1*-1 mutation results in "superinduction" of *RD29A*, *COR47*, *COR15a*, *KIN1*, and *ADH* in response to low temperature (46). Genetic analysis indicates that the *hos1*-1 mutation is recessive. Thus Zhu and colleagues (46) have suggested that the *HOS1* gene encodes a negative regulator of low temperature signal transduction. Interestingly, the *hos1*-1 mutation also results in decreased expression of *COR15a*, *KIN1*, *RAB18*, and *RD29B* in response to ABA, high salt, and high osmoticum (polyethylene glycol). Thus,

the functions of *HOS1* would appear to include a positive role in the induction of at least some genes in response to ABA or osmotic stress (46). Determining the nature of the gene product encoded by *HOS1* should add significantly to an understanding of low temperature gene regulation and, potentially, the interaction of low temperature and ABA signaling pathways.

CONCLUDING REMARKS

Cold acclimation research is in a very exciting phase. Genes and proteins with roles in freezing tolerance are being identified, their mechanisms of action determined, and insights into how the cold-acclimation response is activated in response to low temperature are emerging. In addition, novel strategies for improving plant freezing tolerance are being considered in light of the new results. As discussed by Storlie et al (110), it may be possible to exploit allelic variation at the *Vrn1-Fr1* interval of winter wheat to improve the freezing tolerance of this important crop. Also, as initially alluded to by Artus et al (4), it may be possible to use the *Arabidopsis CBF* genes (or orthologs from other plants) as "master switches" (100) to "manage" activation of freezing tolerance regulons and thereby improve freezing (and possibly drought) tolerance in a broad range of plants. Indeed, it would seem that the next few years promise to bring a burst of fundamental new discoveries regarding the mechanisms and regulation of cold acclimation and efforts to design and evaluate new approaches to improve plant freezing tolerance.

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